Linking behavioral ecology with population genetics: insights from *Drosophila nigrospiracula*

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Although *Drosophila* species provide important model systems for evolutionary biology, the ecologies and natural histories of most species are insufficiently characterized to permit predictions with respect to issues such as population genetic structure. A notable exception is the group of cactophilic *Drosophila* endemic to the Sonoran Desert of North America. One of these species, *D. nigrospiracula*, exhibits no population subdivision anywhere in its range. Here we present evidence suggesting that the timing of mating in relation to dispersal contributes to the panmixia observed in this species.

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Species of the genus *Drosophila* exhibit a wide range of differences in the levels of population genetic differentiation observed using allozyme electrophoresis and molecular genetic studies (Powell 1997; Shoemaker and Jaenike 1997; Markow et al. 2002; Hurtado et al. 2004). An absence of relevant behavioral and ecological studies of the majority of these species makes it difficult to interpret the factors responsible for the presence or absence of population structure. Four species of cactophilic *Drosophila* endemic to the Sonoran Desert of North America, however, have been well characterized with respect to their ecology (Heed 1978, 1982; Breitmeyer and Markow 1998) and behavior (Markow 1982, 1988; Markow and Castrezana 2000). All four species have specialized on different columnar cactus host species, feeding and breeding in necrotic plant tissue or in soil soaked with the necrotic exudates (Heed 1978, 1982). The four *Drosophila* species show contrasting patterns of population subdivision, permitting hypotheses to be tested concerning the relationship between ecology and behavior and population genetic differentiation.

One of the cactophilic species, *D. nigrospiracula* Patterson & Wheeler, exhibits no population structure across its range, compared to the other three desert endemic species, all of which exhibit some degree of local genetic differentiation. These patterns have been found whether the loci used were allozymes (Sluss 1975; Pfeiler and Markow 2001; Markow et al. 2002) or molecular genetic markers (Hurtado et al. 2004), and for *D. nigrospiracula*, gene frequencies have been found to be stable for over 30 years (Sluss 1975; Pfeiler and Markow 2001).

Potential explanations for the observed lack of population structure and the temporal stability of gene frequencies lie in the behavioral ecology of *D. nigrospiracula.* Field studies demonstrate that compared to the other desert *Drosophila*, *D. nigrospiracula* is a strong disperser (Johnston and Heed 1976; Markow and Castrezana 2000). In the laboratory, flies of *D. nigrospiracula* have been found to become sexually mature later than those of *D. melanogaster*: females mate at four and males at six days of age (Markow 1982). Reproductive behavior has been found to differ in other *Drosophila* species, however, when field and laboratory studies have been compared (Markow 1988, 2000; Snook and Markow 2001). Therefore, while it may be tempting to speculate that long distance dispersal coupled with late reproductive maturity could explain the apparent extensive gene flow in *D. nigrospiracula*, caution must be exercised in the absence of information about when flies in nature mate relative to when they disperse and oviposit. For example, if *D. nigrospiracula* mate and oviposit prior to dispersing, adult flies emerging from a given breeding patch would exhibit some degree of inbreeding, as observed in the *Opuntia*-breeding *D. buzzatii* (Barker and Mulley 1976). On the other hand, if flies disperse before they mate, not only should local inbreeding be insignificant, but it would promote a lack of population

structure. These different scenarios can be distinguished by field studies.

Here we ask two questions about *D. nigrospiracula*: 1) when do flies mate relative to when they disperse? 2) is there evidence of inbreeding at a given microhabitat patch? We address these questions through capture-mark-release-recapture studies using virgin flies of different ages and with allozyme electrophoresis of resident adults and newly emerged flies at the same patch.

MATERIAL AND METHODS

Capture-mark-release-recapture experiments

Experiment 1. — This initial experiment was designed to provide an estimate of the proportion of mated females that would be expected to be utilizing a microhabitat patch in the wild and to obtain an estimate of overall recapture rate of marked males and females. Approximately 1450 wild adults of *D. nigrospiracula* of unknown age were collected in January 1997 from a single large necrotic arm of a saguaro cactus (*Carnegiea gigantea*) located at the Superstition study site (Breitmeyer and Markow 1998) east of Tempe, Arizona (33°22′N; 111°22′W). Flies were taken to the laboratory, separated by sex under carbon dioxide anesthesia, counted and then marked with a micronized fluorescent dust (Radiant Corp., Richmond, California) as described in Markow and Castrezana (2000). The following day the marked flies (652 females and 787 males) were released at the same rot from which they were collected. After 24 h the rot was revisited and an exhaustive collection of adult flies was made with a sweep net and by mouth aspiration. We continued collecting until no adult flies could be detected on the rot. Females and males were separated in the field, returned to the laboratory, and scored for the presence of fluorescent dust. Twenty of the recaptured marked females were dissected to check for the presence of sperm in their ventral receptacles.

Experiment 2. — To obtain information on age and mating status of female *D. nigrospiracula*, approximately 2000 flies were reared in the laboratory on potato/saguaro medium (Castrezana 1997) from a mass collection of adults made at the Superstition site in February 1997 at a necrotic saguaro different from that used in experiment 1. Laboratory flies that emerged during a single 24 h period were separated by sex as virgins and stored in bottles with cornmeal and yeast. Approximately 1000 virgin females and 1000 males of known age were thus obtained. The experimental design involved releasing about a third of the females and males each at three different time periods (1, 4 and 7 days post eclosion). In this way females of known age could be recaptured after 24 h and scored for presence of sperm. Flies were marked as before using dusts of different colors for the three different age classes. Females and males were then released on the three different days at a large saguaro rot located at the Superstition site. This rot was different from the one that provided the parental population used in the laboratory mass culture. Twenty-four hours after each release the rot was revisited and flies were collected as before. The color-coded markings revealed that no marked flies from earlier releases co-occurred with those being collected at the time. This suggests that adult flies are quite vagile and do not spend a lot of time at a single rot. Marked females were immediately placed into vials separate from the males so that mating could not occur on transport to the laboratory. Twenty-eight marked females from the day 1 release, and twenty each from the two subsequent releases, were then dissected to check for the presence of sperm. ANOVA was used test if there were significant differences in proportion of flies recaptured by age or sex. Recapture data were arcsine transformed before analysis.

Genetic structure of pre-dispersal emerging flies

Collections of flies. — Wild adults of *D. nigrospiracula* were collected in January 2002 from a single necrotic arm of a cardón cactus (*Pachycereus pringlei*) found at another of our study sites located near Guaymas, Sonora, Mexico (28°00′N; 110°50′W) (Breitmeyer and Markow 1998). After collecting wild flies, a small section of the rotting arm (∼0.5 m in length) was removed and taken to the nearby laboratory facilities at the Instituto Tecnológico y de Estudios Superiores de Monterrey in Guaymas. After determining that no mature wild adults remained on the rot, it was placed into a large covered glass container and was monitored daily for the presence of newly-emerged flies. Immature adults began to appear after about one week. The newly-emerged flies were collected daily over a period of several days and then were separated by sex and placed in 8 dram glass vials containing banana-*Opuntia* culture medium.

Preparation of samples for electrophoresis. — Male and female flies were homogenized individually in 25 µl grinding buffer (10 mM Tris-HCl, 1.0 mM Na_2EDTA, 0.05 mM $NADP^+$; pH 7.5). Homogenates were centrifuged for 2 min at 10 000 g and the supernatants were analyzed by electrophoresis on Titan III cellulose acetate plates (Helena Laboratories, Beaumont, Texas) at room temperature (22°C) for 15–20 min at 200 V using 0.025 M Tris, 0.192 M glycine buffer (pH 8.0). Staining for enzyme activity

followed the method of HEBERT and BEATON (1989) with minor modifications.

The eight enzymes (with abbreviations and EC numbers) analyzed were phosphoglucomutase (PGM; 5.4.2.2) alcohol dehydrogenase (ADH; 1.1.1.1), malate dehydrogenase (MDH; 1.1.1.37), glycerol-3-phosphate dehydrogenase (NAD^+)(GPDH; 1.1.1.8), cytosol non-specific dipeptidase (PEP-A; 3.4.13.18; glycylleucine substrate), tripeptide aminopeptidase (PEP-B; 3.4.11.4; leucylglycylglycine substrate), arginine kinase (ARGK; 2.7.3.3) and carboxylesterase (EST; 3.1.1.1; α-napthylacetate substrate). The loci coding for these enzymes are abbreviated with italics. The common electromorph produced by each locus was used as a reference and was designated 100. Allozymes were assigned numbers according to their relative anodal migration from the origin compared to the reference. Electromorphs migrating to the cathode were designated with a negative sign. Allele frequency data for both sexes were combined for the population analyses (see Results and Discussion).

Statistical analyses. — For statistical analyses, the pre-dispersal newly-emerged flies collected in the laboratory and the wild adults collected at the rot in the field were treated as separate populations. Allele frequency data were imported into BIOSYS (SWOFFORD and SELANDER 1989) for analysis of genetic variation and to test for linkage disequilibrium. LEVENE's (1949) correction for small sample size was used in χ^2 tests for deviation from Hardy-Weinberg equilibrium. FSTAT version 2.9.3 (GOUDET 2001) was used to calculate F-statistics (WEIR and COCKERHAM 1984) and 95% confidence intervals. Pairwise comparison of F_{ST} between the newly emerged and wild flies was performed in FSTAT using 1000 permutations of the data matrix and applying a sequential Bonferroni procedure to correct for multiple comparisons (RICE 1989). F-statistics also were calculated in BIOSYS using both unweighted and weighted mean allele frequencies (NEI 1977; WRIGHT 1978). The resulting overall F-statistics values (not shown) were similar to those from WEIR and COCKERHAM (1984).

RESULTS AND DISCUSSION

Capture-mark-release-recapture

Of the 652 marked females and 787 marked males of *D. nigrospiracula* released at the saguaro rot in the first experiment, 51 females (7.8%) and 70 males (8.9%) were recaptured the next day. The proportions of flies recaptured in this and the following experiment (see below) were similar to values reported in an earlier dispersal study (MARKOW and CASTREZANA 2000). Of the twenty recaptured wild females of unknown age that were dissected, all contained sperm, in agreement with our previous unpublished observations on wild-caught *D. nigrospiracula* which have shown that, to date, effectively all wild-caught females examined are inseminated. This result is consistent with the high frequency of remating noted in the species in the laboratory (MARKOW 1982).

Results from the second experiment are shown in Table 1. Recapture rates (8.6–10.7%) were similar to those in experiment 1. ANOVA indicated no significant differences in proportion of flies recaptured by sex ($F_{1,4}=0.035$; $P=0.861$) or age ($F_{2,3}=0.458$; $P=0.670$). Also, there were no sex-related differences in proportion of recaptured flies among experiments 1 and 2 ($F_{1,6}=0.071$; $P=0.798$). None of the dissected females released on the first day were inseminated, while most, or all, of the older females contained sperm (Table 1). Although we did not test two- and three-day-old females in this study, the results are consistent with laboratory observations showing that females of *D. nigrospiracula* do not begin to mate until about four days after eclosion (MARKOW 1982). Our results also suggest that the youngest flies do not remain more closely associated with their host rot than the older flies. The probability of recapture is similar for flies of different ages suggesting that dispersal rate may be similar over the lifetime of the fly. These results also strongly suggest that females disperse before their first mating. The lack of uninseminated females in wild-caught flies (experiment 1) supports these conclusions, although given the large local population sizes of *D. nigrospiracula* (BREITMEYER and MARKOW 1998), a high proportion of older females at a rot would reduce the likelihood of finding recently-eclosed and uninseminated females in the sample of flies scored for the presence of sperm. If females tend not to mate with males from the original host rot, as our data suggest, the probability of inbreeding at any particular rot is predicted to be low.

Genetic structure of pre-dispersal emerging flies: lack of evidence for inbreeding

Allele frequencies at eight allozyme loci in pre-dispersal immature adults of *D. nigrospiracula* emerging from the cardón rot and wild adults captured at the same rot at the Guaymas study site are shown in Table 2, along with previously unpublished combined data for wild adults collected at the same site during 1998 and 2000 (PFEILER and MARKOW 2001). For wild-caught flies, allele frequencies in the present and previous studies were in close agreement. Of particular interest was the finding that frequencies of the two

Table 1. *Results of the capture-mark-release-recapture experiment (experiment 2) in adult Drosophila nigrospiracula of different ages conducted at the necrotic arm of a saguaro cactus rot at the Superstition site in Arizona.*

Age of flies	Sex	No. released	No. recaptured	(%)	No. recaptured females	
					Dissected	Inseminated
1 day	F	341	30	(8.8)	28	0
	M	319	31	(9.7)		
4 days	F	308	33	(10.7)	20	18
	M	294	26	(8.8)		
7 days	F	292	25	(8.6)	20	20
	M	314	29	(9.2)		

most common alleles in the highly polymorphic *Est-2* locus (alleles 78 and 100) were almost identical in the two studies. Only three (37.5%) of the eight loci examined, *Mdh-1*, *Pep-B* and *Est*, were polymorphic at the 95% level (Table 3). Three loci, *Pgm*, *Adh* and *Gpdh*, were nearly monomorphic as only a single heterozygote for each was found (Table 2). *Argk* was the only locus that was completely monomorphic.

Table 2. *Allele frequencies in adult Drosophila nigrospiracula from Guaymas emerging from a single cardón rot (pre-dispersal) and in wild-caught flies from the same rot (post-dispersal). Combined allele frequencies from wild adults collected from the same locality in 1998 and 2000 are shown in the last column. Number of flies analyzed given in parentheses for each locus.*

Locus/allele	Emerging adults	Wild adults	Wild adults[a]
Pgm	(48)	(48)	(40)
38	0.000	0.010	0.000
100	1.000	0.990	1.000
Adh	(48)	(48)	(60)
−65	0.000	0.000	0.008
−100	1.000	1.000	0.992
Mdh-1	(46)	(48)	(40)
100	0.717	0.833	0.863
131	0.283	0.167	0.137
Gpdh	(48)	(48)	(40)
100	1.000	0.990	1.000
132	0.000	0.010	0.000
Pep-A	(48)	(48)	(40)
90	0.021	0.021	0.013
100	0.979	0.979	0.987
Pep-B	(48)	(48)	(39)
78	0.000	0.000	0.013
83	0.063	0.063	0.115
100	0.916	0.906	0.821
114	0.021	0.021	0.051
120	0.000	0.010	0.000
Argk	(48)	(48)	(24)
100	1.000	1.000	1.000
Est[b]	(47)	(48)	(39)
22	0.043	0.010	0.026
56	0.085	0.063	0.077
78	0.362	0.448	0.423
100	0.415	0.365	0.372
140	0.043	0.031	0.038

[a]from Pfeiler and Markow 2001. [b]frequencies of six rare alleles not shown.

Allele frequencies in freshly-emerged adults were similar to those in wild-caught adults (Table 2). A small difference, however, was observed at the *Mdh-1* locus in pre-dispersal adults of both sexes. Except for *Est-2* in wild adults, all allele frequencies obtained in the present study were in Hardy-Weinberg equilibrium (HWE), including *Pep-B* which was not in HWE in the earlier study (Pfeiler and Markow 2001). The observation that *Est-2* was not in equilibrium is probably an artifact resulting from the high number of alleles at this locus. When all minor alleles of *Est-2* were pooled and compared to the common allele, HWE was found.

Indices of genetic variability (mean heterozygosity, mean number of alleles per locus and percent polymorphic loci) for wild adults of *D. nigrospiracula* from the present and previous studies also were essentially identical (Table 3). In addition, the corresponding indices obtained in recently-emerged flies were very similar to those of wild adults. The value obtained for Nei's (1978) unbiased genetic distance between recently emerged flies and wild adults ($D = 0.001$) also indicated a lack of genetic differentiation between the two groups. This conclusion was supported by a low and statistically insignificant overall F_{ST} value (Table 4). The F_{ST} value at the *Mdh-1* locus, however, indicated a slight, but significant, differentiation among the two groups. No significant linkage disequilibrium ($\alpha = 0.05$) was detected either within or among the two populations. The overall value of F_{IS}, a measure of non-random mating within a subpopulation, was not significant, which is consistent with our prediction that early dispersal in virgin female flies would promote outbreeding. These results are in contrast to those from *D. buzzatii* where inbreeding in flies remaining closely associated with *Opuntia* host

Table 3. *Summary of genetic variability at eight enzyme loci in adult Drosophila nigrospiracula from Guaymas emerging from a single cardón rot (pre-dispersal) and in wild-caught flies collected from the same rot (post-dispersal). Values obtained from wild adults collected from the same locality in 1998 and 2000 are shown in the last row.*

Population	Mean no. alleles per locus (±SE)	% polymorphic loci (95%)	Mean heterozygosity[a]	
			H_O (±SE)	H_E (±SE)
Emerging adults	2.50 (±0.96)	37.5	0.163 (±0.091)	0.163 (±0.091)
Wild adults	2.75 (±0.82)	37.5	0.141 (±0.074)	0.151 (±0.082)
Wild adults[b]	2.63 (±0.84)	37.5	0.142 (±0.083)	0.160 (±0.086)

[a]H_O, observed heterozygosity (direct count); H_E, Hardy-Weinberg expected heterozygosity, unbiased estimate (Nei 1978).
[b]from Pfeiler and Markow 2001.

rots is thought to be one of the factors responsible for the heterozygote deficiencies seen in recently-emerged adults of this species (Barker and Mulley 1976).

When males and females of *D. nigrospiracula* were analyzed separately, pairwise comparisons of F_{ST} between recently-emerged and wild adults also were not significant, but 95% confidence intervals did not overlap [F_{ST} females, −0.018 (95% CI, −0.019 to −0.017); F_{ST} males, 0.023 (95% CI, −0.013 to 0.084)]. Although this can be interpreted as evidence for a sex-specific difference in dispersal (Balloux and Lugon-Moulin 2002), in this case it most likely resulted from the low sample size when the sexes were separated (N = 22–24). Markow and Castrezana (2000) found no evidence for sex-specific differences in dispersal of *D. nigrospiracula*, and our capture-mark-release-recapture data support this conclusion (Table 1). All other indices of genetic variability in the separate analyses of males and females conformed well with the combined data set (not shown) and all allele frequencies for each sex were in HWE.

Table 4. *Summary of F-statistics (Weir and Cockerham 1984) in polymorphic loci in a cohort of pre-dispersal emerging adults of Drosophila nigrospiracula from a single cardón rot and wild-caught adults collected from the same rot in the field.*

Locus	F_{IS}	F_{IT}	F_{ST}
Pgm	0.000	0.000	0.000
Mdh-1	0.073[a]	0.098[a]	0.027[a]
Gpdh	0.000	0.000	0.000
Pep-A	−0.011	−0.021	−0.010
Pep-B	−0.064[a]	−0.074[a]	−0.009
Est-2	0.039	0.037	−0.002
Overall	0.032	0.037	0.005
95% CI	−0.055	−0.064	−0.009
	0.066	0.089	0.024

[a] value falls outside of 95% CI.

In summary, our results, combined with those from previous studies, suggest that stability in genetic variability throughout the geographic range of *D. nigrospiracula* is mediated by a combination of specific behavioral traits, including early dispersal before sexual maturity, frequent remating of longlived females with males from different rots which reduces the potential for inbreeding, and capacity for long distance dispersal.

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